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Folate status and concentrations of serum folate forms in the US population: National Health and Nutrition Examination Survey 2011–2

Christine M. Pfeiffer^{1,*}, Maya R. Sternberg¹, Zia Fazili¹, David A. Lacher², Mindy Zhang¹, Clifford L. Johnson², Heather C. Hamner³, Regan L. Bailey⁴, Jeanne I. Rader⁵, Sedigheh Yamini⁵, R. J. Berry³, and Elizabeth A. Yetley⁴

¹National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA, USA

²National Center for Health Statistics, Centers for Disease Control and Prevention, Hyattsville, MD, USA

³National Center for Birth Defects and Developmental Disabilities, Centers for Disease Control and Prevention, Atlanta, GA, USA

⁴Office of Dietary Supplements, National Institutes of Health, Bethesda, MD, USA

⁵Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, MD, USA

Abstract

Serum and red blood cell (RBC) total folate are indicators of folate status. No nationally representative population data exist for folate forms. We measured serum folate forms [5-methyltetrahydrofolate (5-methylTHF), unmetabolized folic acid (UMFA), non-methyl folate (sum of THF, 5-formylTHF, 5,10-methenylTHF), and MeFox (5-methylTHF oxidation product)] by HPLC-MS/MS and RBC total folate by microbiologic assay in US persons 1 year ($n \sim 7500$) participating in the National Health and Nutrition Examination Survey 2011–2. Data analysis for serum total folate was conducted including and excluding MeFox. Concentrations (geometric mean; detection rate) of 5-methylTHF (37.5 nmol/L; 100%), UMFA (1.21 nmol/L; 99.9%), MeFox (1.53 nmol/L; 98.8%), and THF (1.01 nmol/L; 85.2%) were mostly detectable. 5-FormylTHF (3.6%) and 5,10-methenylTHF (4.4%) were rarely detected. The biggest contributor to serum total folate was 5-methylTHF (86.7%); UMFA (4.0%), non-methyl folate (4.7%), and MeFox (4.5%) contributed smaller amounts. Age was positively related to MeFox but showed a U-shaped pattern for other folates. We generally noted sex and race-ethnic biomarker differences

*Corresponding author: C. M. Pfeiffer, CPfeiffer@cdc.gov.

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and weak (Spearman $r < 0.4$) but significant ($P < 0.05$) correlations with physiologic and lifestyle variables. Fasting, kidney function, smoking, and alcohol intake showed negative associations. BMI and body surface area showed positive associations with MeFox but negative associations with other folates. All biomarkers showed significantly higher concentrations with recent folic acid-containing dietary supplement use. These first-time population data for serum folate forms generally show similar associations with demographic, physiologic, and lifestyle variables as serum total folate. Patterns observed for MeFox may suggest altered folate metabolism dependent on biological characteristics.

Keywords

NHANES; folate vitamers; 5-methyltetrahydrofolate; unmetabolized folic acid; non-methyl folate; folate oxidation product; LC-MS/MS

Introduction

The assessment of folate status has a long tradition in the National Health and Nutrition Examination Survey (NHANES). It has been carried out since 1974 with the use of different measurement procedures and serum and red blood cell (RBC) folate as biomarkers (1). Early measurements were conducted by microbiologic assay (1974–1978), followed by two variants of a radio protein-binding assay (1978–1991 and 1991–2006), and more recently by a much improved microbiologic assay (2007–2010). Folate assays have had continued issues with comparability across laboratories and methods, necessitating the adjustment of data to allow the assessment of trends over time (1–2). The NHANES 2011–2 survey assessed folate status in the US population for the first time by a combination of two analytical methods: serum folate forms were measured by HPLC-MS/MS, while whole-blood folate was measured by microbiologic assay. RBC folate was then calculated using the data from both assays. This approach was the result of a 2010 expert roundtable that advised CDC on folate biomarkers and methods for future NHANES surveys (1). It allows clinicians, public health practitioners, and researchers to obtain information on the full profile of folate forms including unmetabolized folic acid (UMFA). This is important for monitoring purposes in the post-fortification era where an extremely low prevalence of folate deficiency is paired with higher folic acid intake from dietary supplements (3). HPLC-MS/MS is currently the best tool to measure individual folate forms with high sensitivity, specificity, and accuracy (1). While this analytical method produced results fully comparable to those of the microbiologic assay for serum folate, this was not the case for the more complex analysis of whole-blood folate (1). Because these assay differences need to be better understood, HPLC-MS/MS was not used for whole blood folate.

Despite the interest in serum folate forms, currently only information on total folate can be interpreted clinically in the context of folate status. Clearly, the sum of biologically active folate vitamers [5-methyltetrahydrofolate (5-methylTHF), UMFA, tetrahydrofolate (THF), 5-formyltetrahydrofolate (5-formylTHF), and 5,10-methenyltetrahydrofolate (5,10-methenylTHF)] constitutes total folate. But it remains to be determined whether MeFox (pyrazino-s-triazine derivative of 4 α -hydroxy-5-methylTHF) should be included in the

calculation of total folate. This compound is an oxidation product of 5-methylTHF that lacks vitamin biologic activity (4) and is therefore not captured as part of the total folate measurement with the microbiologic assay. While it has been shown that MeFox can be formed *in vitro* after blood collection as a result of suboptimal sample handling (5–6), its possible existence *in vivo* is unclear (5). If the formation of MeFox occurs solely *in vitro* (i.e., after blood collection), then a small part of the formerly active folate pool is lost and MeFox should be included in the total folate calculation to avoid underestimating the biologically active amount of folate. But if some or all of the MeFox may already be present *in vivo* for an extended period of time, including it in the total folate calculation may slightly overestimate the biologically active amount of folate (i.e., folate status will appear better than it is because a small part of the total folate is not biologically available).

Our main objective was to describe serum concentrations of several folate forms in the US population 1 year participating in the NHANES 2011–2 by selected demographic, physiologic, and lifestyle variables. Our second objective was to update information on total folate status and to provide the first nationally representative data for non-Hispanic Asians. We report information on serum total folate with and without the inclusion of MeFox to provide much needed insight on this new topic.

Methods

Participants and study design

The NHANES is conducted by the Centers for Disease Control and Prevention (CDC) and collects cross-sectional data on the health and nutritional status of the civilian non-institutionalized US population by use of a stratified, multistage, probability sample design. In addition to obtaining information in a home interview setting, participants undergo a physical examination and blood draw in a mobile examination center (MEC). In 2011–2, the NHANES oversampled Asian and Hispanic persons in addition to oversampling some other population groups (7–9). The unweighted response rates for participants 1 y of age were 72.2% for the interview component and 69% for the examination component (10). All respondents gave their informed consent, and the NHANES protocol was reviewed and approved by the CDC Research Ethics Review Board.

Biomarker measurement

Serum and whole-blood hemolyate samples from participants 1 year were analyzed by the CDC laboratory for serum folate forms (folate cofactors and MeFox) by use of HPLC-MS/MS (11–13) and for RBC total folate by use of microbiologic assay (14–16), respectively. We did not obtain valid final results for a few samples (<30) resulting in different sample sizes among compounds: serum 5-methylTHF (*n* 7454), UMFA (*n* 7462), THF (*n* 7461), 5-formylTHF (*n* 7466), 5,10-methenylTHF (*n* 7466), MeFox (*n* 7469), and total folate including MeFox (*n* 7442), and RBC total folate (*n* 7867). Sample sizes for the folate biomarkers by covariate categories are presented in Table 1. Because concentrations of the three minor folate forms THF, 5-formylTHF, and 5,10-methenylTHF were often below the limit of detection (LOD) and can be a result of folate interconversions at slightly acidic pH during sample preparation (17), we calculated the sum of these three forms as

non-methyl folate. Serum total folate was calculated as the sum of the six folate forms including MeFox. CDC released results <LOD as imputed values (LOD divided by the square root of two); we used the imputed values in our calculation when the folate form result was <LOD (11). The serum total folate including MeFox result was missing if one of the folate forms was missing. We calculated serum total folate excluding MeFox by subtracting MeFox from total folate including MeFox. RBC total folate was calculated from the measured whole-blood folate concentration after subtracting the serum total folate including MeFox concentration (as determined by HPLC-MS/MS) and adjusting for RBC volume (15). Assay performance is summarized in the online supplementary Table S1 and has been described previously with regard to international reference materials for the HPLC-MS/MS (1,18) and microbiologic assay (14).

Study variables

We categorized the demographic variables as follows: age (1–5, 6–11, 12–19, 20–39, 40–59, and ≥60 years), sex (males and females), and race-ethnicity [Hispanic (Mexican American + other Hispanic), non-Hispanic Asian, non-Hispanic Black, and non-Hispanic White; other racial-ethnic groups were included in overall estimates]. We also reported separate estimates for Mexican Americans to allow comparison to previous reports. We examined physiologic and lifestyle variables previously shown to be associated with folate concentrations (19–20): fasting time (<3, 3–<8, and ≥8 hours), kidney function as determined by estimated glomerular filtration rate [eGFR; 0–<60, 60–<90, and ≥90 mL/(min×1.73 m²)] (21), BMI [<18.5 (underweight), 18.5–<25 (normal), 25–<30 (overweight), and ≥30 kg/m² (obese)] (22), body surface area (BSA, calculated as square root of (height in cm × weight in kg/3600); <1.5, 1.5–<1.8, 1.8–<2.0, and ≥2.0 m²) (23), smoking [serum cotinine ≤10 µg/L (nonsmoker) and >10 µg/L (smoker)] (24), alcohol intake [average daily number of “standard” drinks (1 drink ~15 g alcohol): no drinks, <1 (not 0), 1–<2, and ≥2 drinks/day; only available for participants ≥18 years] (19), and use of folic acid-containing dietary supplements [self-reported use during the 24 hours prior to visiting the MEC (self-reported use during the last 30 days is no longer available in NHANES 2011–2); yes and no]. We presented the association between folate concentrations and body size by two variables: BMI, used traditionally in nutrition studies, and BSA, used in exposure studies to provide insight on “body burden”. A BSA value <1.5 m² generally represents children, while a value ≥2.0 m² generally represents adult men. This additional variable may shed light on the distinction between metabolism vs. “dilution effects” due to body size.

Statistical analysis

We applied no exclusion criteria to our data analysis and used pairwise deletion for missing values in a particular analysis. We used the MEC weights to account for differential nonresponse or noncoverage and to adjust for oversampling of some groups. We calculated the mean percent contribution of each folate form to serum total folate including MeFox. We also calculated the mean absolute and percent contribution of each folate form to serum total folate including MeFox by weighted decile of serum total folate including MeFox. Bivariate associations between geometric mean folate biomarker concentrations (to normalize for right-skewed distributions) and each study variable were described. Geometric means were compared across the categories without (Wald *F* *P* value) and with (Satterthwaite *F* *P* value)

controlling for additional covariates (age, sex, and race-ethnicity). We used Spearman coefficients to assess pairwise correlations among folate biomarkers as well as between folate biomarkers and selected physiologic and lifestyle variables. We assessed the distributions [geometric means and selected percentiles (95% CI)] for each folate biomarker among all participants, fasted (≥ 8 hours) participants, and nonfasted (<8 hours) participants 1 year by demographic variables. Significance was defined as a two-sided P value of <0.05 . Statistical analyses were performed using SAS (version 9, SAS Institute Inc., Cary, NC) and SUDAAN (version 9.2, RTI, Research Triangle Park, NC) software.

Results

A summary of the characteristics of the study population for each variable of interest is given in the online supplementary Table S2. Among US persons 1 year in the unweighted NHANES 2011–2 sample, 27% were children (1–11 years), 14% were adolescents (12–19 years), and 59% were adults (≥ 20 years). Half of the participants were female and almost one-third were non-Hispanic white. Nearly half (43%) of the participants were fasted for ≥ 8 hours, 8% had an impaired eGFR (<60 mL/min/1.73 m²), 24% were obese, 30% had a small BSA (<1.5 m²), 17% were considered smokers (serum cotinine concentrations >10 µg/L), one-third of participants 18 years reported not consuming any alcoholic beverage, and about 20% of participants reported using folic acid-containing dietary supplements during the last 24 hours.

Folate biomarker concentrations and contribution of folate forms to serum total folate

The concentration ranges of serum total folate excluding MeFox, serum total folate including MeFox, and RBC total folate were 3.26–375, 3.50–377, and 149–5490 nmol/L, respectively. Concentration ranges (nmol/L) of serum folate forms were: 1.88–295 for 5-methylTHF, $<\text{LOD}$ (0.14)–282 for UMFA, $<\text{LOD}$ (0.37)–11.5 for THF, $<\text{LOD}$ (0.30)–31.6 for 5-formylTHF, $<\text{LOD}$ (0.34)–4.38 for 5,10-methenylTHF, and $<\text{LOD}$ (0.34)–20.4 for MeFox. Concentrations of 5-methylTHF (100%), UMFA (99.9%), MeFox (98.8%), and THF (85.2%) were detectable in all or most samples, while concentrations of 5-formylTHF (3.6%) and 5,10-methenylTHF (4.4%) were detectable in only a few samples.

On average, 5-methylTHF (86.7%) was the biggest contributor to serum total folate including MeFox, while UMFA (4.0%), non-methyl folate (4.7%), and MeFox (4.5%) contributed smaller amounts. When we calculated the contribution of these folate forms by decile of serum total folate including MeFox (Fig. 1 and online supplementary Table S3), we noted some fluctuation in the proportion of 5-methylTHF (79.9–90.2%), a decreasing proportion of non-methyl folate (9.1% in the first, 2.8% in the last decile) and MeFox (6.6% in the first, 3.0% in the last decile), and a generally U-shaped proportion of UMFA (4.5% in the first, $\sim 3\%$ in the fifth, and 10.4% in the last decile) with increasing decile of serum total folate including MeFox.

Folate biomarker concentrations by demographic characteristics

We noted close to U-shaped age patterns for all serum folate forms except MeFox, for which the concentration was significantly higher in persons ≥ 60 years compared to all other age

groups and the proportion of MeFox relative to serum total folate including MeFox was significantly higher in persons ≥ 60 years compared to the three youngest age groups (Table 2 and online supplementary Fig. 1). Age was a significant factor for all serum folate forms as well as for serum total folate including and excluding MeFox, and RBC total folate whether or not we controlled for other demographic covariates (sex and race-ethnicity). While females had significantly higher serum total folate including and excluding MeFox, RBC total folate, 5-methylTHF, and UMFA concentrations with and without controlling for age and race-ethnicity, there were no sex differences for non-methyl folate and MeFox concentrations. All folate biomarker concentrations except non-methyl folate varied significantly by race-ethnicity, with non-Hispanic whites having the highest concentrations and non-Hispanic Asians having similar concentrations compared to Hispanics. These observations did not change after we controlled for age and sex. The concentration difference between geometric means of serum total folate excluding vs. including MeFox was ~ 2 nmol/L and it was reasonably consistent across demographic groups.

Correlations among folate biomarkers

Spearman correlations were significant for most pairwise comparisons of folate biomarkers in persons ≥ 1 year (see online supplementary Table S4). We observed significant and strong ($r \geq 0.7$) correlations for 5-methylTHF with serum total folate including MeFox ($r = 0.99$) and for THF with non-methyl folate ($r = 1.0$). We observed significant and moderate ($0.4 \leq r < 0.7$) correlations for 5-methylTHF with RBC total folate ($r = 0.59$), for UMFA with 5-methylTHF ($r = 0.44$) and serum total folate including MeFox ($r = 0.50$), and for serum total folate including MeFox with RBC total folate ($r = 0.59$). We observed significant but weak ($r < 0.4$) correlations for 5-methylTHF with MeFox ($r = 0.25$). After stratifying by age group, we noted a strengthening of the correlations in persons ≥ 60 years (trace covered a larger area than for persons 1–19 or 20–59 years), but the same patterns overall (Fig. 2).

Associations between folate biomarkers and selected physiologic and lifestyle characteristics

We observed generally significant but weak Spearman correlations between folate biomarkers and the continuous physiologic and lifestyle variables (Table 3). Fasting, kidney function, smoking, and alcohol intake were negatively associated with most folate biomarkers. BMI and body surface area showed positive associations with MeFox and negative associations with other folates. We noted generally significant differences in concentrations between the levels of the categorical variables, including categorized versions of the physiologic and lifestyle variables, whether or not we controlled for demographic covariates (age, sex, and race-ethnicity) (Table 4). All folate biomarkers showed significantly higher concentrations with recent folic acid-containing dietary supplement use whether or not we controlled for demographic covariates. The concentration difference between serum total folate excluding vs. including MeFox was ~ 2 nmol/L and it was again reasonably consistent across categories of variables.

Reference intervals and distributions of folate biomarker concentrations

Because fasting was a significant factor for most folate biomarkers, we calculated the central 95% reference intervals (2.5th – 97.5th percentile) for all, fasted, and nonfasted “generally healthy” persons 1 year (Table 5). Reference intervals were fairly comparable among these three groups for 5-methylTHF, non-methyl folate, serum total folate excluding and including MeFox, and RBC total folate. However, we noted a lower upper end of the reference interval in fasted persons for UMFA and MeFox.

Selected percentiles (5th to 95th) presented by age, sex, and race-ethnicity for all, fasted, and nonfasted persons 1 year generally showed the greatest variation by age group (see online supplementary Tables S5–S11). We observed distinct differences in the distributions of serum total folate excluding and including MeFox, RBC total folate, and 5-methylTHF by age group. We also observed differences in the upper end of the distribution of UMFA by age group, higher non-methyl folate concentrations at the lower end of the distribution for children 1–5 years compared to any other age group, and a right-shift in the distribution of MeFox with increasing age group. The differences we observed in the central 95% reference intervals between all and fasted persons were also notable in the entire distribution of folate concentrations.

Folate status time trend

Serum total folate excluding MeFox concentrations [geometric mean (95% CI)] were similar in 2011–2 [41.4 (40.1–42.9) nmol/L] compared to the previous two survey cycles, when the microbiologic assay was used, which does not respond to MeFox: 2007–8 [39.5 (37.7–41.3) nmol/L] and 2009–0 [38.2 (37.2–39.3) nmol/L]. RBC total folate concentrations measured in all three survey cycles by microbiologic assay also appeared to be similar: 2007–8 [1120 (1070–1160) nmol/L], 2009–0 [1040 (1010–1070) nmol/L], and 2011–2 [1050 (1010–1090) nmol/L]. As in the previous two survey cycles, <1% of the US population in NHANES 2011–2 had serum (<10 nmol/L) or RBC total folate (<340 nmol/L) concentrations at risk for deficiency (25).

Discussion

This study provides the first national reference information for serum folate forms measured by HPLC-MS/MS in a population exposed to folic acid fortification. It also offers a better understanding of variables associated with concentrations of serum folate forms. Based on the newest serum and RBC total folate concentrations from NHANES 2011–2, the folate status of the US population was comparable to previous years and non-Hispanic Asians had similar folate concentrations compared to Hispanics.

Previous studies that assessed the profile of serum folate forms used convenience samples and were small in size (mostly <100 subjects). Most studies investigated special population subgroups such as pregnant women, older adults, or hemodialysis patients (26–30), while a few studies measured serum folate forms in apparently healthy US, German, or Norwegian adults, though generally as part of method validations (5,17,31–34). Given that the population in this study was exposed to folic acid fortification and known to have a

historical prevalence of folic acid supplement use of ~35% (35), the higher 5-methylTHF (38.5 nmol/L) and UMFA (0.991 nmol/L) median concentrations compared to the small convenience sample reports for German [15.8 nmol/L and 0.10 nmol/L, respectively, (34)] or Norwegian [16.4 nmol/L and 0.0 nmol/L, respectively, (54)] adults from countries with no folic acid fortification were not surprising. However, caution should be used when comparing data from different populations, in part due to potential method differences that have historically plagued folate analyses (36).

The 5-methylTHF concentration was the biggest and a constant contributor to serum total folate regardless of the population [86.7% in US (this study) compared to 87.2% in German (34) and 85.8% in Norwegian persons (5)]. The higher mean UMFA concentration (13.5 nmol/L) and relative contribution (10.2%) in the highest decile of serum total folate including MeFox compared to the lower deciles (0.78–2.87 nmol/L, 2.75–4.45%) in this study is likely due to the larger intake of folic acid from dietary supplements and/or fortified foods and the incomplete conversion of folic acid to 5-methylTHF upon absorption (37–39). A previous report from NHANES 2007–8 showed that UMFA concentrations >1 nmol/L were largely explained by total folic acid intake from diet and supplements apart from fasting status (40). Not surprisingly, we found significantly higher serum folate forms as well as serum and RBC total folate concentrations in persons who reported consuming folic acid-containing dietary supplements during the last 24 hours.

Given that MeFox is an oxidation product of 5-methylTHF, the correlation between these two folate forms ($r = 0.25$) was lower than expected. This may indicate that factors beyond the amount of circulating 5-methylTHF may influence the generation of MeFox. Thus, the relevance of MeFox in relation to folate status is likely of interest in any population, regardless of whether they have high folate status as a result of fortification or supplementation or not. The high correlations between 5-methylTHF and serum total folate ($r = 0.99$) and between THF and non-methyl folate ($r = 1.00$) were expected, as these two folate forms were the major contributors to serum total folate and non-methyl folate, respectively. We found a correlation between UMFA and 5-methylTHF ($r = 0.54$ for persons ≥ 60 y) similar to that reported for older German adults ($r = 0.42$ at baseline and $r = 0.56$ after supplementation with folic acid) (30). We found lower correlations between UMFA and THF ($r = 0.22$) or between 5-methylTHF and THF ($r = 0.30$) in US older persons compared to the report in German older adults (at baseline: $r = 0.39$ and $r = 0.51$, respectively; after supplementation: $r = 0.45$ and $r = 0.56$, respectively) (30).

Among demographic variables studied, we found interesting patterns with age. While most folate forms displayed the typical U-shaped age pattern previously documented with serum and RBC total folate (41), concentrations of MeFox showed a linear pattern and were highest in persons ≥ 60 years. The distribution of MeFox concentrations showed a right-shift with increasing age group resulting in higher detection rates of MeFox in persons ≥ 60 years (99.7% compared to 95.5% in children 1–5 years). Conversely, detection rates of THF were highest in children 1–5 years (96.1% compared to 82.9–89.1% for other age groups). These observations may indicate altered folate metabolism, possibly as a result of aging, and will have to be confirmed in other studies. It is interesting though to note that older age was

associated with less bioactive folate (THF) and more biologically inactive folate (MeFox), possibly pointing to an increased catabolism.

The associations of serum folate forms with physiologic and lifestyle variables were generally consistent with reports for serum total folate from NHANES 2003–6 (19–20). While most serum folate forms showed higher concentrations in nonfasted persons, we found no difference in RBC total folate concentrations by fasting time, consistent with the prevailing knowledge that RBC total folate concentrations are not affected by fasting (42). The negative association of serum folate forms and of serum and RBC total folate with decreasing kidney function was no longer significant for several folate biomarkers (5-methylTHF, non-methyl folate, total folate excluding and including MeFox) after we controlled for demographic covariates. Most serum folate forms showed lower concentrations in smokers and in persons with higher alcohol intake.

Some observations with physiologic and lifestyle variables, particularly for MeFox, were unexpected and may indicate altered folate metabolism, possibly challenging the notion that MeFox occurs solely *in vitro*. BMI and BSA were positively associated with MeFox, but negatively with 5-methylTHF concentrations. This is counterintuitive if one assumes that higher 5-methylTHF concentrations will lead to higher MeFox concentrations. It may in fact indicate a greater “loss” of active folate in obesity. Whether this has any bearing on epidemiologic observations linking obesity with a higher rate of neural tube defects (43) is unclear, particularly if one considers that in our study obese vs. normal weight persons had ~5.5 nmol/L lower 5-methylTHF, but only ~0.2 nmol/L higher MeFox concentrations. We need a better understanding of these relationships to appropriately interpret them. The lower serum total folate (and higher RBC total folate) concentrations reported previously with higher BMI led to the hypothesis that cellular uptake and tissue distribution of folate may be altered by BMI (44). The current data by BSA category may instead imply a “dilution effect” whereby most folate forms and serum total folate concentrations were lower in larger persons. This interpretation was also consistent with the finding that children <11 years had higher 5-methylTHF and serum total folate concentrations compared to all other age groups.

We found a median MeFox concentration (1.49 nmol/L) similar to that reported in Norwegian adults [2.3 nmol/L; the authors called this compound 4 α -hydroxy-5-methylTHF but stated that it was the pyrazino-s-triazine derivative, (5)], suggesting that concentrations of this oxidation product are low as long as the blood is processed under controlled conditions. Yet, we found apparent inconsistencies regarding MeFox and 5-methylTHF concentrations (Table 5) which raised the possibility that MeFox may be formed *in vivo* rather than solely *in vitro* as part of 5-methylTHF oxidation. In rats, administration of radio-labeled folic acid resulted in the excretion of several labeled products: a precursor of MeFox (4- α -hydroxy-5-methylTHF), 5-methylTHF, 10-formyl-folic acid, and *p*-aminobenzoyl-L-glutamate, a folate breakdown product (45). It is therefore conceivable that a small portion of MeFox measured in serum may already be in circulation at the time of the blood draw. We know that MeFox can be formed during the pre-analytical phase (blood collection and processing) (5–6), leading to a small loss of “active” folate after blood is collected. We have shown that the analytical phase of the CDC LC-MS/MS method does not generate additional MeFox (11). Thus, it appears that failure to include MeFox as part of serum total folate may

slightly underestimate folate status if MeFox is mostly formed *in vitro*, while including it may slightly overestimate folate status if MeFox is mostly formed *in vivo*. Mechanistic studies that further explore the origins of this oxidation product are needed.

Our study is subject to some limitations. The data are based on only one NHANES survey period limiting our ability to generalize findings for some stratifications. While we evaluated the association of folate biomarkers with recent use of folic acid-containing dietary supplements as part of selected lifestyle factors, evaluating dietary folate intake overall and according to intake sources (fortified cereal-grain foods, ready-to-eat cereals, supplements, and combinations thereof) was beyond the scope of this study. Lastly, because these are the first national estimates of folate vitamers, we are limited in our ability to compare findings to other studies of similar magnitude.

In summary, these novel data on serum folate forms generally show associations between these compounds and selected demographic, physiologic, and lifestyle variables similar to those reported previously for serum total folate. However, particularly for MeFox, we observed distinct patterns with the variables studied that may suggest altered folate metabolism dependent on biological characteristics. Thus, measuring MeFox as part of the folate profile may provide relevant information in populations with high or low folate status. While we cannot as yet answer the question of whether it is more accurate to include or exclude MeFox from the total folate, the difference between the two approaches is rather small (~5%). Based on the findings of this study and for practical reasons we suggest that until an unequivocal answer is found, MeFox should NOT be included in the calculation of serum total folate, but should be separately reported to allow an assessment of the quality of sample handling as well as potential insight into folate metabolism. This approach has two advantages: the total folate without MeFox can be directly compared to the microbiologic assay and other assays that do not measure this biologically inactive form and one errs on the side of caution with the interpretation of folate status by slightly underestimating it. The new reference intervals for serum folate forms in a population that has been exposed to folic acid fortification for over 15 years provide a much-needed benchmark to researchers and public health officials in those nations in which folic acid fortification has already occurred (e.g., the United States, Canada) and where folic acid intakes are significant contributors to total folate intakes, but also to nations that consider folic acid fortification (e.g., the United Kingdom).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

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|-------------------------|---|
| 5-formylTHF | 5-formyltetrahydrofolate |
| 5,10-methenylTHF | 5,10-methenyltetrahydrofolate |
| 5-methylTHF | 5-methyltetrahydrofolate |
| BSA | body surface area |
| CDC | Centers for Disease Control and Prevention |
| eGFR | estimated glomerular filtration rate |
| LOD | limit of detection |
| MeFox | pyrazino-s-triazine derivative of 4 α -hydroxy-5-methylTHF |
| MEC | Mobile Examination Center |
| NHANES | National Health and Nutrition Examination Survey |
| RBC | red blood cell |
| THF | tetrahydrofolate |
| UMFA | unmetabolized folic acid |

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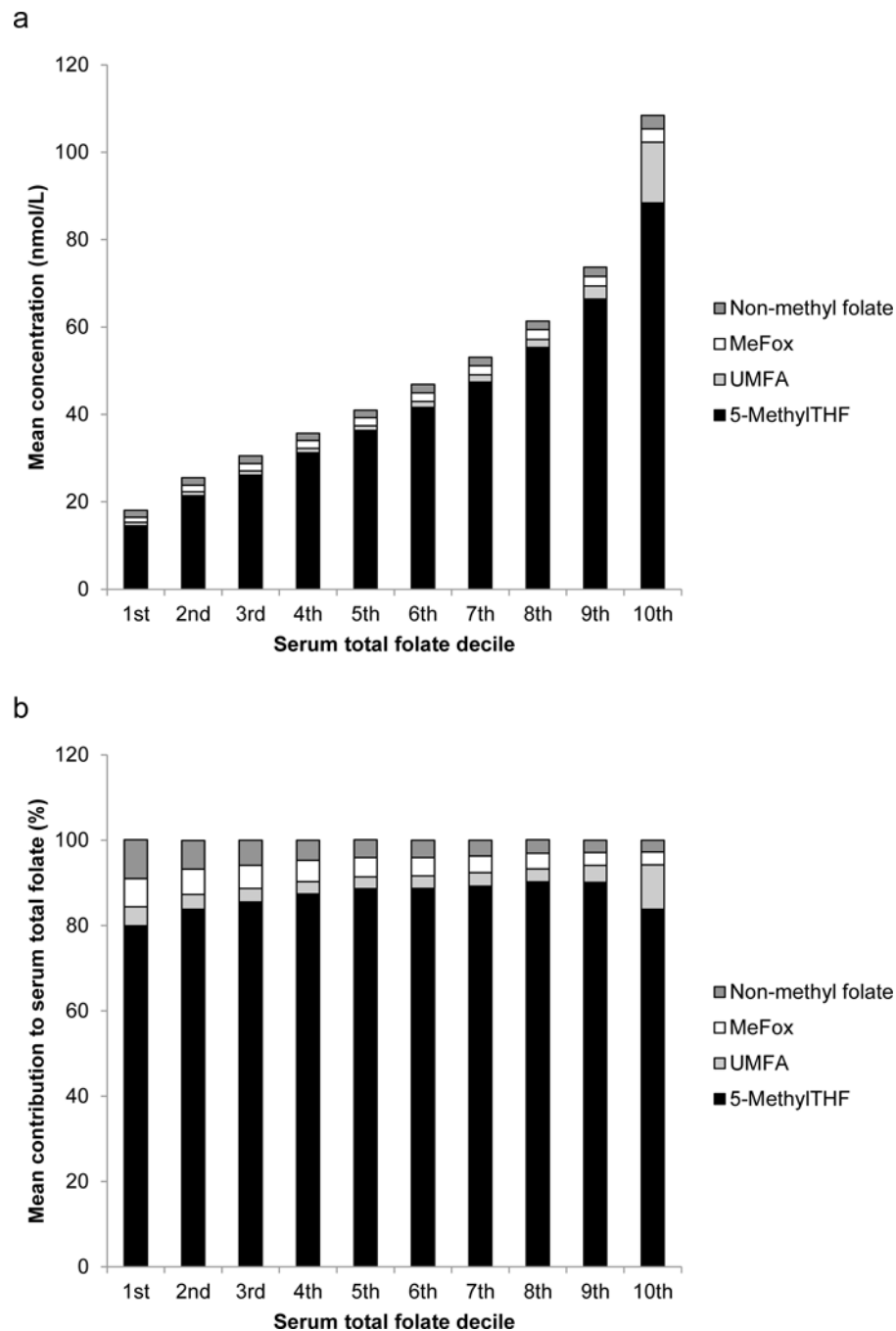


Figure 1.

Mean absolute (panel a) and relative (panel b) contribution of folate forms to serum total folate by weighted decile of serum total folate in the US population 1 year, NHANES 2011–2. Serum folate forms and serum total folate (sum of folate forms including MeFox) were measured by HPLC-MS/MS. Non-methyl folate represents the sum of three minor forms tetrahydrofolate, 5-formyltetrahydrofolate and 5,10-methenyltetrahydrofolate. 5-MethylTHF, 5-methyltetrahydrofolate; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF; UMFA, unmetabolized folic acid.

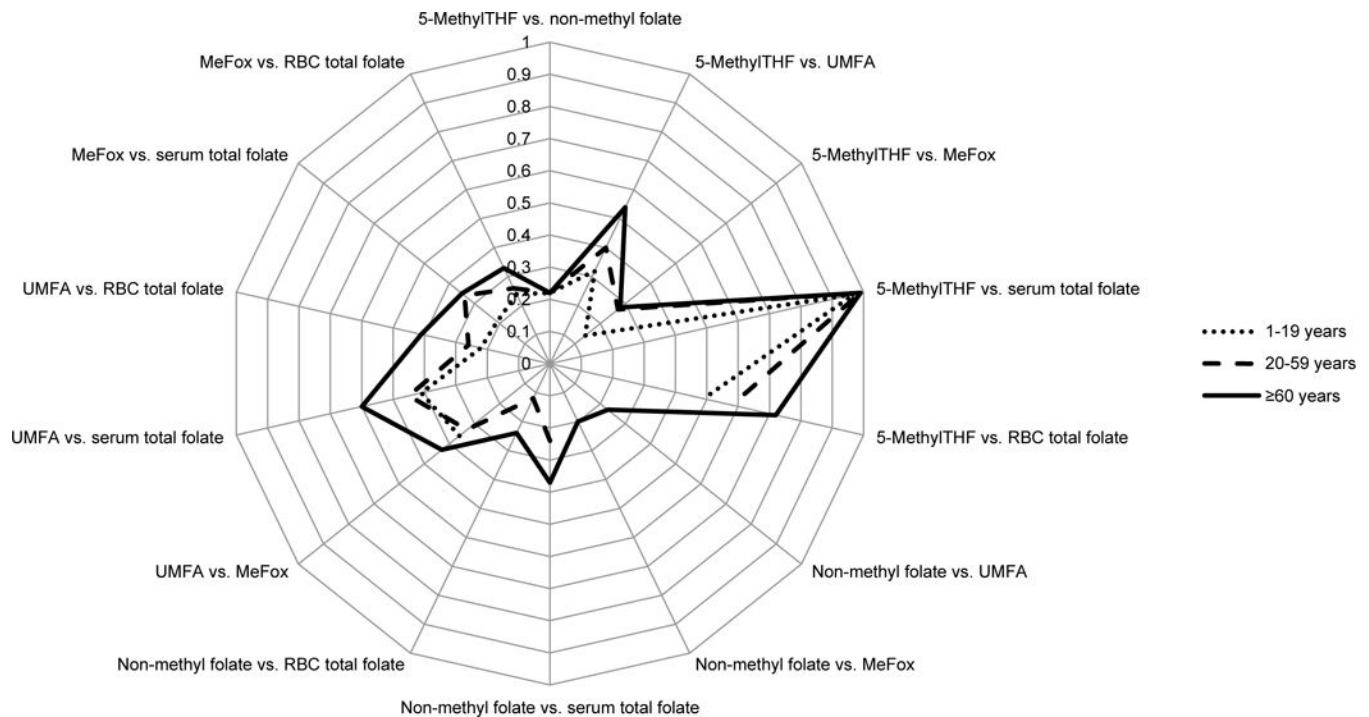


Figure 2.

Spearman correlation between various folate biomarkers by age group in the US population

1 y, NHANES 2011–2. Only statistically significant correlations are shown (see supplementary Table S4 for complete information). Serum folate forms and serum total folate (sum of folate forms including MeFox) were measured by HPLC-MS/MS. Non-methyl folate represents sum of three minor forms: tetrahydrofolate, 5-formyltetrahydrofolate and 5,10-methenyltetrahydrofolate. RBC total folate was measured by microbiologic assay. 5-MethylTHF, 5-methyltetrahydrofolate; FOL, folate; MeFox, pyrazino-s-triazine derivative of 4 α -hydroxy-5-methylTHF; RBC, red blood cell; UMFA, unmetabolized folic acid.

Table 1

Unweighted sample sizes for serum folate forms and serum and red blood cell total folate by selected demographic, physiologic, and lifestyle variable categories for the US population 1 year, NHANES 2011–2

| Variable categories | 5-MethylTHF | UMFA | Serum Non-methyl folate | MeFox | Total folate | RBC Total folate |
|---|-------------|------|----------------------------|-------|--------------|---------------------|
| All | 7454 | 7462 | 7458 | 7469 | 7442 | 7867 |
| Age, year | | | | | | |
| 1–5 | 568 | 573 | 570 | 575 | 563 | 708 |
| 6–11 | 955 | 957 | 959 | 960 | 953 | 1044 |
| 12–19 | 1073 | 1074 | 1074 | 1074 | 1073 | 1127 |
| 20–39 | 1730 | 1730 | 1730 | 1730 | 1730 | 1762 |
| 40–59 | 1604 | 1604 | 1605 | 1606 | 1603 | 1655 |
| 60 | 1524 | 1524 | 1520 | 1524 | 1520 | 1571 |
| Sex | | | | | | |
| Male | 3739 | 3745 | 3746 | 3750 | 3735 | 3944 |
| Female | 3715 | 3717 | 3712 | 3719 | 3707 | 3923 |
| Race-ethnicity ^b | | | | | | |
| Hispanic | 1798 | 1803 | 1803 | 1806 | 1794 | 1919 |
| Mexican American | 989 | 993 | 995 | 996 | 987 | 1071 |
| Other Hispanic | 809 | 810 | 808 | 810 | 807 | 848 |
| Non-Hispanic Asian | 947 | 947 | 948 | 951 | 944 | 999 |
| Non-Hispanic Black | 2025 | 2027 | 2027 | 2027 | 2025 | 2185 |
| Non-Hispanic White | 2401 | 2402 | 2398 | 2402 | 2397 | 2471 |
| Other | 283 | 283 | 282 | 283 | 282 | 293 |
| Fasting time, hours | | | | | | |
| <3 | 2476 | 2479 | 2475 | 2482 | 2469 | 2644 |
| 3–<8 | 1633 | 1637 | 1635 | 1637 | 1631 | 1744 |
| 8 | 3345 | 3346 | 3348 | 3350 | 3342 | 3479 |
| eGFR stage ^c , mL/(min×1.73 m ²) | | | | | | |
| 0–<60 | 467 | 467 | 466 | 467 | 466 | 466 |
| 60–<90 | 2032 | 2032 | 2031 | 2033 | 2030 | 2039 |
| 90 | 3382 | 3383 | 3383 | 3384 | 3381 | 3428 |

| Variable categories | 5-MethylTHF | UMFA | Serum Non-methyl folate | MeFox | Total folate | RBC Total folate |
|--|-------------|------|----------------------------|-------|--------------|---------------------|
| BMI ^d | | | | | | |
| Underweight | 1203 | 1206 | 1204 | 1209 | 1197 | 1382 |
| Normal weight | 2297 | 2301 | 2301 | 2303 | 2295 | 2415 |
| Overweight | 1854 | 1854 | 1855 | 1855 | 1854 | 1882 |
| Obese | 1921 | 1921 | 1918 | 1921 | 1918 | 1970 |
| BSA ^e , cm ² ×kg | | | | | | |
| <1.5 | 1756 | 1762 | 1761 | 1766 | 1750 | 1971 |
| 1.5–1.8 | 1989 | 1990 | 1989 | 1991 | 1987 | 2069 |
| 1.8–2 | 1596 | 1596 | 1596 | 1597 | 1595 | 1640 |
| 2 | 1934 | 1934 | 1932 | 1934 | 1932 | 1969 |
| Serum cotinine ^f , µg/L | | | | | | |
| 10 | 5938 | 5941 | 5937 | 5945 | 5929 | 6068 |
| >10 | 1240 | 1240 | 1240 | 1241 | 1239 | 1259 |
| Alcohol intake ^g | | | | | | |
| No drinks | 1474 | 1474 | 1472 | 1474 | 1472 | 1530 |
| <1 (not 0) | 2540 | 2540 | 2539 | 2541 | 2538 | 2602 |
| 1–<2 | 342 | 342 | 342 | 342 | 342 | 349 |
| 2 | 235 | 235 | 234 | 235 | 234 | 243 |
| Supplement use ^h | | | | | | |
| Yes | 1303 | 1305 | 1302 | 1306 | 1299 | 1370 |
| No | 5567 | 5573 | 5574 | 5579 | 5561 | 5882 |

NHANES, National Health and Nutrition Examination Survey; 5-methylTHF, 5-methyltetrahydrofolate; UMFA, unmetabolized folic acid; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF; RBC, red blood cell; eGFR, estimated glomerular filtration rate; BSA, body surface area.

^a Serum folate forms and serum total folate (sum of all forms including MeFox) were measured by HPLC-MS/MS; RBC total folate was measured by microbiologic assay; non-methyl folate represents sum of three minor forms: tetrahydrofolate, 5-formyltetrahydrofolate, and 5,10-methylenetetrahydrofolate.

^b Hispanic subgroup represents sum of Mexican American and other Hispanic ethnicity; other subgroup includes persons with multi-ethnic background.

^c Used to assess renal function; available for persons 12 years; impaired renal function was defined as eGFR <60 mL/(min×1.73 m²)

^d BMI definitions (kg/m²): <18.5 (underweight); 18.5–>25 (normal weight); 25–<30 (overweight); and 30 (obese)

^e Calculated as square root of [(height in cm × weight in kg)/3600] or square root of [(height in inches × weight in pounds)/3131]

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Biomarker of tobacco smoke exposure; concentrations $>10 \mu\text{g/L}$ are considered to represent smokers
Calculated for participants 18 years as average daily number of “standard” drinks [(quantity \times frequency)/365.25]; 1 drink $\approx 15 \text{ g ethanol}$
Folic acid-containing dietary supplements used during the last 24 hours prior to visiting the Mobile Examination Center

Table 2

Concentrations of serum folate forms and serum and red blood cell total folate by demographic variable categories for the US population 1 year, NHANES 2011–2^a

| Variable | 5-MethylTHF | UMFA | Non-methyl folate | Serum | | Total folate without MeFox | Total folate with MeFox | RBC | |
|-----------------------------|------------------|------------------|-------------------|------------------|----------|----------------------------|-------------------------|------------------|--------------|
| | | | | MeFox | (nmol/L) | | | Total folate | Total folate |
| All | 37.5 (36.0–39.1) | 1.21 (1.15–1.28) | 1.59 (1.32–1.92) | 1.53 (1.45–1.61) | | 41.4 (40.1–42.9) | 43.4 (42.0–44.9) | 1050 (1010–1090) | |
| Age, year | | | | | | | | | |
| 1–5 | 53.6 (50.5–57.0) | 1.72 (1.46–2.02) | 1.92 (1.68–2.18) | 1.31 (1.12–1.52) | | 59.5 (56.1–63.2) | 61.2 (57.7–65.0) | 1030 (985–1070) | |
| 6–11 | 53.2 (50.3–56.4) | 1.55 (1.36–1.78) | 1.75 (1.44–2.13) | 1.39 (1.27–1.51) | | 58.5 (55.4–61.8) | 60.4 (57.2–63.7) | 1060 (1020–1090) | |
| 12–19 | 37.1 (34.8–39.5) | 1.10 (1.02–1.17) | 1.44 (1.18–1.75) | 1.33 (1.19–1.48) | | 40.5 (38.4–42.7) | 42.2 (39.9–44.5) | 933 (899–969) | |
| 20–39 | 32.0 (30.6–33.3) | 1.04 (.968–1.12) | 1.55 (1.22–1.96) | 1.41 (1.32–1.51) | | 35.5 (34.2–36.8) | 37.3 (36.0–38.6) | 961 (922–1000) | |
| 40–59 | 34.3 (32.3–36.4) | 1.14 (1.04–1.24) | 1.49 (1.25–1.78) | 1.51 (1.43–1.60) | | 38.1 (36.0–40.3) | 40.0 (37.8–42.3) | 1060 (1000–1110) | |
| 60 | 44.4 (42.1–46.8) | 1.49 (1.36–1.64) | 1.77 (1.49–2.11) | 1.98 (1.86–2.11) | | 49.0 (46.7–51.5) | 51.7 (49.3–54.2) | 1260 (1200–1330) | |
| P value | <0.0001 | <0.0001 | <0.0001 | <0.0001 | | <0.0001 | <0.0001 | <0.0001 | |
| P value adjusted | <0.0001 | <0.0001 | 0.0002 | <0.0001 | | <0.0001 | <0.0001 | <0.0001 | |
| Sex | | | | | | | | | |
| Male | 35.8 (34.0–37.6) | 1.17 (1.09–1.25) | 1.57 (1.30–1.90) | 1.50 (1.40–1.60) | | 39.6 (38.0–41.2) | 41.5 (39.8–43.2) | 1030 (982–1070) | |
| Female | 39.2 (37.6–40.9) | 1.26 (1.18–1.34) | 1.60 (1.33–1.94) | 1.55 (1.48–1.64) | | 43.3 (41.7–45.1) | 45.4 (43.7–47.2) | 1070 (1040–1110) | |
| P value | 0.0002 | 0.0438 | 0.15 | 0.18 | | 0.0004 | 0.0005 | 0.0040 | |
| P value adjusted | 0.0001 | 0.0349 | 0.25 | 0.23 | | 0.0002 | 0.0002 | 0.0039 | |
| Race-ethnicity ^b | | | | | | | | | |
| Hispanic | 36.1 (34.3–38.0) | 1.01 (.924–1.12) | 1.73 (1.26–2.38) | 1.26 (1.20–1.33) | | 40.0 (38.1–42.0) | 41.7 (39.8–43.6) | 961 (937–984) | |
| MA | 37.4 (34.8–40.2) | .984 (.872–1.11) | 1.78 (1.28–2.47) | 1.21 (1.13–1.29) | | 41.2 (38.9–43.5) | 42.8 (40.5–45.1) | 973 (940–1010) | |
| NH Asian | 37.2 (35.4–39.1) | .945 (.864–1.03) | 1.60 (1.28–2.00) | 1.59 (1.45–1.74) | | 40.9 (39.1–42.8) | 43.1 (41.2–45.1) | 952 (911–994) | |
| NH Black | 29.8 (28.2–31.4) | 1.19 (1.12–1.26) | 1.83 (1.35–2.46) | 1.21 (1.16–1.26) | | 34.0 (32.2–35.8) | 35.6 (33.8–37.4) | 860 (830–892) | |
| NH White | 39.4 (38.0–40.9) | 1.30 (1.23–1.37) | 1.52 (1.29–1.79) | 1.66 (1.59–1.74) | | 43.3 (41.9–44.9) | 45.5 (44.0–47.0) | 1130 (1080–1170) | |
| P value | <0.0001 | <0.0001 | 0.35 | <0.0001 | | <0.0001 | <0.0001 | <0.0001 | |
| P value adjusted | <0.0001 | <0.0001 | 0.21 | <0.0001 | | <0.0001 | <0.0001 | <0.0001 | |

NHANES, National Health and Nutrition Examination Survey; 5-methylTHF, 5-methyltetrahydrofolate; UMFA, unmetabolized folic acid; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF; RBC, red blood cell; MA, Mexican American; NH, non-Hispanic.

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Values are geometric means (95% CI); serum folate forms and serum total folate (sum of folate forms excluding or including MeFox) were measured by HPLC-MS/MS; RBC total folate was measured by microbiologic assay; non-methyl folate represents sum of three minor forms: tetrahydrofolate, 5-formyltetrahydrofolate, and 5,10-methylenetetrahydrofolate; for sample sizes, see Table 1; P value is the unadjusted Wald F p-value, while P value adjusted is the Satterthwaite F P value adjusted for age, sex, and race-ethnicity.

Hispanic subgroup represents the sum of Mexican American and other Hispanic ethnicity; P values for race-ethnicity show comparison of Hispanic, NH White, NH Black, NH Asian, and other (not shown).

Spearman correlation between various folate biomarkers and selected physiologic and lifestyle variables for the US population 1 year, NHANES 2011–2012^a

Table 3

| Variable categories | Serum | | | | RBC | |
|-----------------------------|-------------|---------|-------------------|---------|----------------------------|-------------------------|
| | 5-MethylTHF | UMFA | Non-methyl folate | MeFox | Total folate without MeFox | Total folate with MeFox |
| Fasting time | | | | | | |
| Spearman <i>r</i> | −0.02 | −0.19 | 0.01 | −0.34 | −0.04 | −0.06 |
| <i>P</i> value | 0.10 | <0.0001 | 0.39 | <0.0001 | 0.0093 | 0.0005 |
| eGFR ^b | | | | | | |
| Spearman <i>r</i> | −0.07 | −0.17 | −0.07 | −0.23 | −0.07 | −0.08 |
| <i>P</i> value | <0.0001 | <0.0001 | 0.07 | <0.0001 | <0.0001 | <0.0001 |
| BMI ^c | | | | | | |
| Spearman <i>r</i> | −0.22 | −0.08 | −0.02 | 0.08 | −0.22 | −0.22 |
| <i>P</i> value | <0.0001 | 0.0021 | 0.31 | <0.0001 | <0.0001 | <0.0001 |
| BSA ^d | | | | | | |
| Spearman <i>r</i> | −0.27 | −0.10 | −0.05 | 0.04 | −0.27 | −0.26 |
| <i>P</i> value | <0.0001 | 0.0005 | 0.0241 | 0.0040 | <0.0001 | <0.0001 |
| Serum cotinine ^e | | | | | | |
| Spearman <i>r</i> | −0.25 | −0.14 | −0.02 | −0.06 | −0.25 | −0.24 |
| <i>P</i> value | <0.0001 | <0.0001 | 0.71 | 0.0097 | <0.0001 | <0.0001 |
| Alcohol intake ^f | | | | | | |
| Spearman <i>r</i> | −0.10 | −0.14 | −0.04 | −0.13 | −0.10 | −0.11 |
| <i>P</i> value | 0.0022 | <0.0001 | 0.05 | <0.0001 | 0.0022 | 0.0012 |

NHANES, National Health and Nutrition Examination Survey; 5-methylTHF, 5-methyltetrahydrofolate; UMFA, unmetabolized folic acid; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF; RBC, red blood cell; eGFR, estimated glomerular filtration rate; BSA, body surface area.

^a Serum folate forms and serum total folate (sum of folate forms excluding or including MeFox) were measured by HPLC-MS/MS; RBC total folate was measured by microbiologic assay; non-methyl folate represents sum of three minor forms: tetrahydrofolate, 5-formyl-tetrahydrofolate, and 5,10-methylenetetrahydrofolate; for sample sizes, see Table 1

^b Used to assess renal function; available for persons 12 years; impaired renal function was defined as eGFR <60 mL/(min×1.73 m²)

^c BMI definitions (kg/m²): <18.5 (underweight); 18.5–>25 (normal weight); 25–<30 (overweight); and 30 (obese)

^d Calculated as square root of [(height in cm × weight in kg)/3600] or square root of [(height in inches × weight in pounds)/3131]

f Calculated for participants 18 years as average daily number of "standard" drinks [(quantity \times frequency)/365.25]; 1 drink \approx 15 g ethanol

Concentrations of serum folate forms and serum and red blood cell total folate by selected physiologic and lifestyle variables for the US population year, NHANES 2011–2^a

| Variable categories | 5-Methyl/THF | UMFA | Serum | | Total folate without MeFox | Total folate with MeFox | RBC Total folate |
|---|------------------|------------------|-------------------|------------------|----------------------------|-------------------------|---------------------|
| | | | Non-methyl folate | MeFox | | | |
| | | | (nmol/L) | | | | |
| Fasting time, hours | | | | | | | |
| <3 | 39.1 (37.5–40.7) | 1.52 (1.44–1.61) | 1.57 (1.31–1.88) | 1.93 (1.84–2.03) | 43.7 (42.2–45.3) | 46.2 (44.6–47.8) | 1040 (1000–1080) |
| 3–<8 | 37.8 (35.8–40.0) | 1.40 (1.29–1.52) | 1.66 (1.37–2.00) | 1.96 (1.83–2.10) | 42.2 (40.1–44.4) | 44.8 (42.5–47.1) | 1050 (988–1120) |
| 8 | 36.3 (34.7–37.9) | .978 (.923–1.04) | 1.57 (1.29–1.92) | 1.17 (1.10–1.25) | 39.6 (38.2–41.1) | 41.1 (39.6–42.7) | 1060 (1020–1090) |
| <i>P</i> value | <0.0001 | <0.0001 | 0.19 | <0.0001 | <0.0001 | <0.0001 | 0.25 |
| <i>P</i> value adjusted | 0.0108 | <0.0001 | 0.44 | <0.0001 | 0.0170 | 0.0006 | 0.43 |
| eGFR ^b , mL/(min×1.73 m ²) | | | | | | | |
| 0–<60 | 42.3 (39.1–45.8) | 1.74 (1.57–1.92) | 1.80 (1.46–2.22) | 2.89 (2.66–3.15) | 47.5 (43.8–51.4) | 51.3 (47.6–55.2) | 1330 (1240–1430) |
| 60–<90 | 36.5 (34.8–38.3) | 1.24 (1.15–1.33) | 1.57 (1.31–1.89) | 1.61 (1.54–1.68) | 40.4 (38.7–42.2) | 42.5 (40.7–44.3) | 1100 (1060–1140) |
| 90 | 34.6 (33.0–36.3) | 1.05 (.988–1.13) | 1.52 (1.24–1.88) | 1.37 (1.26–1.48) | 38.1 (36.6–39.7) | 39.9 (38.3–41.5) | 982 (939–1030) |
| <i>P</i> value | 0.0001 | <0.0001 | 0.0406 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| <i>P</i> value adjusted | 0.48 | 0.0006 | 0.26 | <0.0001 | 0.42 | 0.11 | 0.0019 |
| BMI ^c | | | | | | | |
| Underweight | 49.3 (46.3–52.4) | 1.62 (1.43–1.83) | 1.76 (1.49–2.07) | 1.36 (1.22–1.52) | 54.7 (51.7–57.8) | 56.6 (53.5–59.7) | 1010 (982–1040) |
| Normal weight | 38.8 (36.9–40.8) | 1.21 (1.14–1.30) | 1.55 (1.28–1.88) | 1.47 (1.36–1.59) | 42.8 (41.0–44.8) | 44.7 (42.8–46.8) | 1010 (973–1050) |
| Overweight | 36.7 (35.4–38.2) | 1.21 (1.14–1.29) | 1.55 (1.28–1.87) | 1.52 (1.44–1.61) | 40.5 (39.2–41.9) | 42.4 (41.0–43.9) | 1060 (1020–1110) |
| Obese | 33.3 (31.6–35.0) | 1.09 (.998–1.18) | 1.61 (1.30–1.99) | 1.65 (1.56–1.76) | 36.9 (35.2–38.6) | 39.0 (37.3–40.8) | 1090 (1040–1150) |
| <i>P</i> value | <0.0001 | 0.0002 | 0.0048 | 0.0001 | <0.0001 | <0.0001 | 0.0068 |
| <i>P</i> value adjusted | 0.0004 | 0.0015 | 0.73 | 0.0046 | 0.0004 | 0.0006 | 0.0026 |
| BSA ^d , cm×kg | | | | | | | |
| <1.5 | 50.2 (47.8–52.8) | 1.55 (1.39–1.72) | 1.78 (1.50–2.10) | 1.41 (1.31–1.52) | 55.5 (53.1–58.0) | 57.4 (54.9–60.0) | 1040 (1020–1060) |
| 1.5–1.8 | 38.5 (36.2–40.9) | 1.23 (1.15–1.32) | 1.56 (1.28–1.90) | 1.53 (1.43–1.64) | 42.6 (40.2–45.1) | 44.6 (42.2–47.2) | 1020 (975–1060) |
| 1.8–2 | 36.0 (33.9–38.1) | 1.15 (1.09–1.22) | 1.55 (1.27–1.89) | 1.53 (1.43–1.63) | 39.6 (37.5–41.8) | 41.6 (39.4–43.8) | 1050 (995–1110) |
| 2 | 33.0 (31.3–34.8) | 1.11 (1.02–1.20) | 1.56 (1.29–1.89) | 1.57 (1.48–1.67) | 36.6 (35.0–38.2) | 38.6 (36.9–40.2) | 1080 (1030–1140) |
| <i>P</i> value | <0.0001 | <0.0001 | 0.0019 | 0.0009 | <0.0001 | <0.0001 | 0.24 |

| Variable categories | 5-MethylTHF | UMFA | Non-methyl folate | Serum | | Total folate without MeFox | Total folate with MeFox | RBC | |
|------------------------------------|------------------|-------------------|-------------------|------------------|--|----------------------------|-------------------------|------------------|--|
| | | | | MeFox | | | | Total folate | |
| <i>P</i> value adjusted | 0.0053 | 0.0131 | 0.63 | 0.0159 | | 0.0036 | 0.0040 | 0.0309 | |
| Serum cotinine ^e , µg/L | | | | | | | | | |
| 10 | 39.6 (37.8–41.4) | 1.25 (1.19–1.32) | 1.60 (1.33–1.93) | 1.54 (1.46–1.63) | | 43.6 (42.0–45.3) | 45.6 (43.9–47.4) | 1080 (1040–1130) | |
| >10 | 29.3 (27.6–31.2) | 1.04 (0.97–1.17) | 1.52 (1.22–1.89) | 1.49 (1.38–1.61) | | 32.9 (31.3–34.7) | 34.8 (33.1–36.7) | 928 (877–981) | |
| <i>P</i> value | <0.0001 | 0.0025 | 0.26 | 0.24 | | <0.0001 | <0.0001 | <0.0001 | |
| <i>P</i> value adjusted | <0.0001 | 0.0052 | 0.68 | 0.22 | | <0.0001 | <0.0001 | <0.0001 | |
| Alcohol intake ^f | | | | | | | | | |
| No drinks | 38.2 (36.6–40.0) | 1.33 (1.25–1.42) | 1.68 (1.36–2.06) | 1.81 (1.73–1.89) | | 42.5 (40.6–44.4) | 44.8 (42.9–46.8) | 1090 (1030–1140) | |
| <1 (not 0) | 35.7 (33.9–37.6) | 1.18 (1.09–1.27) | 1.54 (1.26–1.89) | 1.53 (1.44–1.61) | | 39.5 (37.7–41.4) | 41.4 (39.6–43.4) | 1060 (1010–1100) | |
| 1–2 | 31.7 (28.0–35.9) | 1.06 (0.949–1.18) | 1.60 (1.34–1.91) | 1.45 (1.30–1.61) | | 35.3 (31.6–39.5) | 37.2 (33.3–41.4) | 1110 (981–1250) | |
| 2 | 30.5 (27.6–33.6) | .855 (.732–.998) | 1.52 (1.19–1.93) | 1.35 (1.14–1.59) | | 33.6 (30.7–36.7) | 35.3 (32.4–38.5) | 1010 (940–1080) | |
| <i>P</i> value | 0.0021 | 0.0003 | 0.14 | <0.0001 | | 0.0018 | 0.0010 | 0.28 | |
| <i>P</i> value adjusted | 0.0162 | 0.0002 | 0.57 | 0.0017 | | 0.0108 | 0.0074 | 0.39 | |
| Supplement use ^g | | | | | | | | | |
| Yes | 52.9 (51.0–54.9) | 2.10 (1.92–2.29) | 1.78 (1.49–2.13) | 1.79 (1.67–1.92) | | 59.2 (56.9–61.5) | 61.5 (59.2–63.9) | 1360 (1290–1430) | |
| No | 33.6 (32.1–35.2) | 1.02 (.964–1.09) | 1.53 (1.26–1.87) | 1.44 (1.37–1.52) | | 37.0 (35.7–38.4) | 38.9 (37.5–40.4) | 973 (940–1010) | |
| <i>P</i> value | <0.0001 | <0.0001 | 0.0043 | <0.0001 | | <0.0001 | <0.0001 | <0.0001 | |
| <i>P</i> value adjusted | <0.0001 | <0.0001 | 0.0008 | 0.0028 | | <0.0001 | <0.0001 | <0.0001 | |

NHANES, National Health and Nutrition Examination Survey; 5-methylTHF, 5-methyltetrahydrofolate; UMFA, unmetabolized folic acid; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF; RBC, red blood cell; eGFR, estimated glomerular filtration rate; BSA, body surface area.

^aValues are geometric means (95% CI); serum folate forms and serum total folate (sum of folate forms excluding or including MeFox) were measured by HPLC-MS/MS; RBC total folate was measured by microbiologic assay; non-methyl folate represents sum of three minor forms: tetrahydrofolate, 5-formyl-tetrahydrofolate, and 5,10-methylenetetrahydrofolate; for sample sizes, see Table 1; *P* value is the unadjusted Wald *P*-value, while *P* value adjusted is the Satterthwaite *F* *P* value adjusted for age, sex, and race-ethnicity.

^bUsed to assess renal function; available for persons 12 years; impaired renal function was defined as eGFR <60 mL/(min×1.73 m²)

^cBMI definitions (kg/m²): <18.5 (underweight); 18.5–25 (normal weight); 25–30 (overweight); and 30 (obese)

^dCalculated as square root of [(height in cm × weight in kg)/3600] or square root of [(height in inches × weight in pounds)/3131]

^eBiomarker of tobacco smoke exposure; concentrations >10 µg/L are considered to represent smokers

^fCalculated for participants 18 years as average daily number of “standard” drinks [(quantity × frequency)/365.25]; 1 drink ≈ 15 g ethanol

^gFolic acid-containing dietary supplements used in the last 24 hours prior to visiting the Mobile Examination Center

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Central 95% reference intervals for serum folate forms and for serum and red blood cell total folate in the US population 1 year, NHANES 2011–2^a

Table 5

| Analyte | All persons | | | Fasted persons | | | Nonfasted persons | | |
|-------------------------|------------------|------------------|----------|------------------|------------------|--|-------------------|------------------|--|
| | 2.5th | 97.5th | | 2.5th | 97.5th | | 2.5th | 97.5th | |
| | | | (nmol/L) | | | | | | |
| Serum 5-methylTHF | 12.3 (11.4–13.2) | 95.4 (92.0–99.8) | | 11.7 (10.8–12.5) | 96.1 (91.4–104) | | 13.2 (11.6–13.9) | 94 (91.5–96.6) | |
| Serum UMFA | .410 (.368–.449) | 17.2 (14.3–21.0) | | .383 (.341–.416) | 4.93 (2.99–9.70) | | .450 (.397–.480) | 28.0 (219–37.7) | |
| Serum non-methyl folate | <LOD | 5.94 (5.31–7.53) | | <LOD | 5.89 (5.06–7.69) | | <LOD | 6.08 (5.50–7.62) | |
| THF | <LOD | 5.38 (4.74–7.16) | | <LOD | 5.37 (4.55–7.37) | | <LOD | 5.39 (4.83–7.34) | |
| 5-FormylTHF | <LOD | .340 (<LOD–.392) | | <LOD | .336 (<LOD–.406) | | <LOD | .343 (.317–.383) | |
| 5,10-MethenylTHF | <LOD | .478 (.414–.570) | | <LOD | .480 (–) | | <LOD | .629 (.532–.775) | |
| Serum MeFox | .445 (.401–.475) | 6.13 (5.70–6.77) | | .384 (<LOD–.424) | 4.75 (4.20–5.55) | | .536 (.496–.565) | 7.01 (6.66–7.59) | |
| Serum total folate | | | | | | | | | |
| Excluding MeFox | 14.7 (13.6–15.9) | 107 (103–112) | | 14.1 (13.3–14.8) | 103 (98.6–111) | | 16.0 (14.5–17.0) | 111 (104–124) | |
| Including MeFox | 16.0 (14.5–16.9) | 109 (106–115) | | 14.7 (13.8–16.0) | 105 (100–113) | | 17.1 (16.4–18.0) | 114 (108–126) | |
| RBC total folate | 477 (460–493) | 2320 (2200–2520) | | 476 (445–505) | 2340 (2250–2480) | | 477 (460–492) | 2300 (2100–2560) | |

NHANES, National Health and Nutrition Examination Survey; 5-methylTHF, 5-methyltetrahydrofolate; UMFA, unmetabolized folic acid; THF, tetrahydrofolate; 5-formylTHF, 5-formyltetrahydrofolate; 5,10-methenylTHF, 5,10-methenyltetrahydrofolate; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF; RBC, red blood cell

^aValues are percentiles (95% CI); serum folate forms and serum total folate (sum of folate forms excluding or including MeFox) were measured by HPLC-MS/MS; RBC total folate was measured by microbiologic assay; non-methyl folate represents sum of three minor forms: THF, 5-formylTHF, and 5,10-methenylTHF; for sample sizes, see Table 1.

Table 6

Inconsistencies between serum MeFox and 5-methyltetrahydrofolate concentrations in the US population 1 year, NHANES 2011–2

| Variable | Finding |
|---|---|
| Correlation between MeFox and 5-methylTHF | Unexpectedly low ($r = 0.25$) considering that MeFox is an oxidation product of 5-methylTHF |
| Age | MeFox concentrations seemed to increase linearly with age while 5-methylTHF concentrations showed a U-shaped age pattern |
| Sex | MeFox concentrations did not differ by sex while 5-methylTHF concentrations were higher in females |
| Fasting status | MeFox concentrations were much lower in fasted (> 8 hours) compared to nonfasted (<3 hours) persons (39%) while 5-methylTHF concentrations were only a little bit lower (7%); the central 95% reference intervals for serum total folate excluding and including MeFox were almost the same in fasted persons, but not in nonfasted persons |
| Kidney function | MeFox concentrations were much higher (111%) in persons with poor (eGFR <60 mL/(min \times 1.73 m ²) compared to good (eGFR \geq 90 mL/(min \times 1.73 m ²) kidney function while 5-methylTHF concentrations were only a little bit higher (22%) |
| BMI | MeFox concentrations were higher (21%) with higher BMI (obese vs. underweight) while 5-methylTHF concentrations were lower (32%) |
| BSA | MeFox concentrations were higher (11%) with higher BSA (> 2 vs. <1.5 cm ² /kg) while 5-methylTHF concentrations were lower (34%) |
| Smoking status | MeFox concentrations did not differ by smoking status while 5-methylTHF concentrations were lower (26%) in smokers |

MeFox, pyrazino-s-triazine derivative of 4 α -hydroxy-5-methylTHF; NHANES, National Health and Nutrition Examination Survey; 5-methylTHF, 5-methyltetrahydrofolate; eGFR, estimated glomerular filtration rate; BSA, body surface area.